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Fine Structural Study of the Cortical Reaction and Formation of the Egg Coats in a Lancelet (= Amphioxus), *Branchiostoma floridae* (Phylum Chordata: Subphylum Cephalochordata = Acrania)

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Abstract. A method for artificial fertilization of lancelet eggs is described, and the egg coats are studied for the first time by transmission electron microscopy. Large, ovarian oocytes and spawned, unfertilized eggs (which are about 140 μm in diameter) are surrounded by a coarsely granular vitelline layer about 1 μm thick and a jelly layer a few micrometers thick. The egg cortex is crowded with a monolayer of cortical granules, each with an average diameter of approximately 3.5 μm. About 20 to 30 s after insemination, a cortical reaction occurs almost simultaneously over the entire egg surface. The cortical granules undergo exocytosis, and part of their content evidently forms a dense layer 30 nm thick against the inside of the vitelline layer: both layers together constitute the fertilization envelope, which begins elevating from the egg surface. By 80 s after insemination, the jelly layer has disappeared, and beneath the fertilization envelope the bulk of the ejected cortical granule material has become organized into a hyaline layer with a finely fibrogranular consistency. By 20 min after insemination, the perivitelline space between the fertilization envelope and the egg surface has attained its maximum width of roughly 150 μm, and both the hyaline layer and the vitelline layer component of the fertilization envelope are much attenuated and remain so until hatching about 9 h after insemination. Egg coats are compared among major deuterostome groups, and the results imply that the ancestral chordate may have been an unspecialized appendicularian.

Introduction

Lancelets, when first discovered in the 1770's, were incorrectly placed in the phylum Mollusca; however, when rediscovered in the 1830's, they were classified without any controversy as primitive fishes (Drach, 1948). In 1865 and 1867, when Kowalevsky described lancelet development, his essential points were that the later embryology and larval development are vertebrate-like, but the early embryology is much like that of an invertebrate deuterostome. These results soon influenced the evolutionary ideas of the time, as discussed by Drach (1948), Dawydoff (1961), and Vucinich (1988). In the mid 1860's, von Baer, one of the world's most respected embryologists, was convinced that there were three groups of invertebrates and one group of vertebrates with no genetic continuity among groups. According to this theory of limited transformationism, there was an unbridgeable gap in the chain of being between the invertebrates and the vertebrates. Kowalevsky's work on lancelet embryology put a quick end to von Baer's notion of limited transformationism and did much to help establish Darwin's conception of a single tree of life. In addition, Kowalevsky ignited a passion for comparative embryology that gripped the German universities until the end of the nineteenth century.

In spite of the impact of Kowalevsky's lancelet work on the history of science, neither he nor the many lancelet biologists who came after him ever established exactly how lancelets are related to the other major groups of animals. Roughly a dozen conflicting schemes for lancelet phylogeny have been proposed, many of which have been reviewed by Haeckel (1893), Drach (1948), Bone (1960), Hennig (1983), and Jefferies (1986). New information should at least help one to discard the most unlikely of these hypotheses, and we have sought to discover useful new character states in the very early development of lancelets.

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Until now, technical problems have forced students of lancelet development to start their descriptions with stages no earlier than the already fertilized egg. Artificial fertilization of lancelet eggs *in vitro* has proven difficult, having previously been accomplished only by Tung *et al.* (1958), who limited their descriptions to blastomere fates and did not concern themselves with earlier development. In other studies of the subject, it has been necessary to initiate lancelet development by allowing males to spawn together with females, either at the time of collection in the field (Kowalevsky, 1865, 1867, 1877; Hatschek, 1882, 1893; Wilson, 1893; Sobotta, 1895, 1897; Van der Stricht, 1896; Cerfonatine, 1906; Conklin, 1932, 1933; Bone, 1958) or in captivity (Lwoff, 1892; Orton, 1913–1915; Webb, 1958; Flood, 1975).

Recently, while studying *Branchiostoma floridae*, a lancelet found along coasts of the southeastern USA, we discovered how to induce spawning in the laboratory. Thus we have been able to investigate the earliest events of development, including sperm-egg interactions, the cortical reaction, the formation of the egg coats, and rearrangements of the egg cytoplasm. The chief purpose of the present paper is to describe the closely related phenomena of the cortical reaction and the formation of the egg coats. The descriptions are based on transmission electron microscopy (TEM) and light microscopy (LM) in conjunction with videotape recording. Surprisingly, this is the first TEM study of any developmental stage of a lancelet younger than the two-week old larva (Flood, 1975). Although our coverage of the first minutes of development is not intended to be comprehensive, we will give context to our work by including a TEM description of the investments of the large oocytes within the ovary and by touching upon polar body emission. The discussion includes a comparison of egg coats between lancelets and other major groups of deuterostome animals.

Materials and Methods

Adult males and females of *Branchiostoma floridae* (Hubbs) (formerly *B. caribaeum*) were collected by shovel and sieve from sandy substratum in water about 1 m deep approximately 100 m south of the Courtney Campbell Causeway in Old Tampa Bay, Florida. After transport to the nearby University of South Florida, the lancelets were distributed individually in dishes of filtered seawater from the field (27‰) at laboratory room temperature (25° ± 1°C). The sexes of the ripe animals were distinguishable because the yellow ovaries or white testes were visible through the body wall. All collections were made during the last week of August and the first week of September, 1988, a time of year when the Tampa Bay population of *B. floridae* was at its ripest according to Nelson (1968); in spite of this, only about 20% of the lancelets in any given collection had gonads of maximum size. Importantly, no males or females spawned while being collected, transported, or maintained in the laboratory—unless we stimulated them as described below.

During the evening of the day of collection, ripe females were stimulated to spawn with a non-lethal electric shock. Each female, lying in a dish of seawater about 2 cm deep, was placed between two platinum electrodes connected to an electronic stimulator (Grass Instrument Co., Quincy, Massachusetts) and shocked for 2 s with 200 direct current pulses each of 10 ms and 50 volts. Since the electric current produced some chlorine electrolytically, the animal was transferred to uncontaminated seawater immediately after shocking. Of about 100 ripe females stimulated, 14 spawned. Approximately 5 min elapsed between stimulation and spawning, which lasted for a few minutes. During each spawning episode, a few hundred to a few thousand eggs were emitted from the atrial pore. Although many of the anterior and posterior gonads were not emptied by spawning, additional electric shocks elicited a second spawning in only two of the females. Once spawned, the eggs remained fertilizable for at least 2 h.

Motile sperm were obtained from the ripe male lancelets by a method so reliable that we did not attempt to induce males to spawn electrically. Because our preliminary results showed that sperm excised from ripe testes were usually immotile in seawater, we induced motility by dissecting the testes in seawater that contained 10 m*M* NH_4Cl and had been adjusted to pH 8 with NaOH. Clouds of motile sperm exiting from the cut testis could be collected by pipette at a concentration roughly equivalent to a 1:500 dilution of dry sperm. Our unpublished TEM showed that this method of inducing motility did not trigger the acrosome reaction.

For insemination, two drops of sperm suspension were added with rapid mixing to approximately 10^3 eggs in 3 ml of filtered seawater. This relatively high concentration of sperm, which fortunately resulted in little polyspermy, helped to ensure simultaneous fertilization of all the eggs. After 30 min, the embryos were transferred to 8 ml of filtered seawater in a petri dish (5 cm in diameter), and thereafter the seawater in the culture dish was renewed at hourly intervals. Depending on the egg batch, a moderate to high proportion of the embryos developed normally, and ciliated neurulae hatched after 9 to 10 h at a temperature of 25° ± 1°C.

TEM fixation, rinsing, and postfixation were by Holland's (1988) glutaraldehyde-dichromate procedure. These solutions were relatively hypertonic to minimize the enormous swelling of the cortical granules that occurred in less concentrated TEM solutions (our unpub. obs.). We fixed large oocytes (contained in fragments of

ripe ovaries), spawned eggs, and developmental stages at the following intervals after insemination: 20 s, 40 s, 80 s, 2 min, 4 min, 6 min, 8 min, 10 min, 15 min, 20 min, 40 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h. After postfixation, all specimens were dehydrated in an ethanol series and embedded in Spurr's resin. Contrast of silver sections was enhanced with uranyl acetate and lead citrate.

Elevation of the fertilization envelope was recorded on videotape via a video camera (Panasonic model WV3170) attached to a compound microscope. Each egg was inseminated uncompressed in a drop (0.15 ml) of seawater on a microscope slide. From continuous videotape records of six eggs, egg diameters and the elevation of the fertilization envelope were measured every 10 s for 20 min with a stop-frame video cassette recorder (Panasonic model NV8950). All measurements were calibrated with a stage micrometer that had been videotaped with the same apparatus. An additional 37 eggs were measured at 20 min after insemination to determine their diameters and the maximum elevation of their fertilization envelopes.

Results

In Table I, our terminology for the egg investments of *B. floridae* is correlated with that in the earlier literature on lancelet reproduction. In two instances, we created names for structures seen by us for the first time. Otherwise, previously established names have been retained as much as possible. However, since a few of the older terms imply incorrect homologies with structures in other organisms (see discussion), we have substituted more appropriate names.

Investments of large oocytes in the ovary (TEM Fig. 1)

The largest germinal cells within the ovary are primary oocytes, which are generally called ovarian eggs in the older literature. Our TEM has clarified the relationship of the oocyte to the surrounding ovarian tissues, which are the squamous epithelium of the primary ovarian cavity (defined by Zarnik, 1904) and the underlying ovarian blood sinus. The blood sinus is filled with plasma comprising closely packed granules of moderate density, each having a diameter of approximately 15 nm. There is a basal lamina but no endothelium delimiting the ovarian blood sinus, which thus has a fine structure comparable to the rest of the circulatory system in lancelets (Rähr, 1981). When the lancelet oocyte is ovulated into the secondary ovarian cavity (as described by Zarnik, 1904), both the epithelium of the primary ovarian cavity and the blood sinus remain behind as an integral part of the ovarian tissues.

Ovarian oocytes approaching maximal size have a dented surface topography somewhat resembling that of a golf ball. Two extracellular matrices intervene between the oocyte plasma membrane and the blood sinus of the ovary. The matrix closer to the oocyte is the vitelline layer, which is about 0.8 μm thick and consists of dense, oblong granules (about 20×60 nm). Short microvilli on the oocyte surface project into the vitelline layer. The more distant matrix is the jelly layer, which is up to 3 μm thick in each surface dent of the oocyte. The jelly consists of loosely packed, fine fibrils, each about 3 nm in diameter. Although our TEM revealed no obvious stages in the synthesis or exocytosis of either matrix by the oocyte, the follicle epithelium, being squamous and located on the outer side of the blood sinus, would not appear to be the source of these matrices. Instead, it is likely that the oocyte itself synthesizes and secretes the jelly layer and the vitelline layer (both of which would thus be primary egg envelopes as defined by Wourms, 1987).

Spawned, unfertilized eggs (TEM Figs. 2–4)

The spawned eggs are, *sensu stricto,* secondary oocytes, having become arrested at the second meiotic metaphase at about the time of ovulation (Van der Stricht, 1896). In *B. floridae,* the living spawned eggs are spherical and their diameters range from 115 to 158 μm, with an average of 138 μm (SD = 9.2; n = 43). The surface dents of the ovarian oocyte have smoothed out, and numerous microvilli about 1 μm long project from the surface of the spawned egg and penetrate most of the thickness of the vitelline layer, which is now 1.2 μm thick. The jelly layer, which is at most about 1.5 μm thick in TEM preparations, is located just outside the vitelline layer. The jelly forms a continuous layer around some eggs, but is more patchily distributed on the surface of others. Evidently the jelly layer is not much thicker in the living state, because no obvious spaces separate living eggs crowded in a monolayer and observed by LM.

The cortex of the unfertilized egg is dominated by a monolayer of cortical granules each of which is ovoid to pyriform (averaging 2.5 μm wide $\times$ 4.5 μm long) with its long axis perpendicular to the overlying plasma membrane (Fig. 2). The bounding membrane of the granule is usually broken in many places in our TEM preparations due to some swelling of the contents, which consist of a packed mass of 30 nm granular or fibrillar material of relatively low electron density. Most of the cortical granules are separated from the overlying plasma membrane by no more than about 50 nm of intervening cytoplasm. The cytoplasmic regions separating adjacent cortical granules in the egg cortex contain abundant free ribosomes, some profiles of endoplasmic reticulum and a very few mitochondria. This cortical cytoplasm (Fig. 3) typically appears denser than the deeper cytoplasm (Fig. 4) in our electron micrographs, possibly due to the un-

Table I

Investments of lancelet eggs: comparison of terms used in the present and previous studies (Italian, German, and French terms have been translated literally into English)

LARGE OOCYTE IN OVARY

Present study (TEM)	Zarnik, 1904 (LM)	Reverberi ([illegible]), 1971 (TEM)	Reverberi and De Leo, 1972 (TEM)	Guraya, 1978, 1983 (LM)
Epithelium of primary ovarian cavity	Follicle epithelium	External follicle	Cellular envelope	Thecal layer
Blood sinus	Blood sinus	Internal follicle	Gelatinous substance	Basal lamina
Jelly layer	No mentioned	Not mentioned	Not mentioned	Not mentioned
Vitelline layer	Not mentioned	Niche material	Granular sheet	Zona pellucida

UNFERTILIZED EGG

Present study (TEM)	Sobotta, 1897 (LM)	Cerfontaine, 1906 (LM)
Jelly layer	Not mentioned	Not mentioned
Vitelline layer	External membrane	Vitelline membrane

FERTILIZED EGG

Present study (TEM)	Sobotta, 1897 (LM)	Several 19th century references (LM)[1]	Cerfontaine, 1906 (LM)	Several 20th century references (LM)[2]	Recent reviews[3]
Jelly layer	Not mentioned	Not mentioned	Not mentioned	Not mentioned	Not mentioned
Fertilization envelope	External membrane	Vitelline membrane	Vitelline membrane	Fertilization membrane	Fertilization membrane
Hyaline layer	Internal membrane (= Chief membrane)	Not mentioned	Perivitelline membrane	Not mentioned	Jelly-like substance

[1] Kowalevsky, 1867; Hatschek, 1882; Willey, 1894; Van der Stricht, 1896.
[2] Conklin, 1932; Tung *et al.*, 1958; Wickstead, 1973.
[3] Reverberi, 1971; Guraya, 1983.

avoidable swelling of the cortical granules during TEM processing. The cytoplasm beneath the cortical zone includes numerous ribosomes, many mitochondria and yolk granules. The yolk granules, which range in diameter from about 2 to 5 μm, have a complicated structure including fibrillar components that Song and Wu (1986) have previously described by TEM for the yolk granules of ovarian oocytes in *B. belcheri*. The TEM of the remaining cytoplasmic and chromosomal strutures is beyond the scope of the present study.

Elevation of the fertilization envelope (LM, Figs. 5–8)

About 20 to 30 s after insemination, the elevation of the fertilization envelope begins (the TEM of this structure will be described in the next section of the results), and a perivitelline space opens up between the envelope and the plasma membrane of the egg. In some eggs, elevation begins a few seconds sooner at the animal pole (conveniently marked by the presence of the first polar body) than elsewhere on the egg surface; however, in many eggs, elevation begins simultaneously over the entire surface. Continuous videotape records of six eggs (Fig. 5) show that the rate of elevation was greatest in the first few minutes after insemination and that the first polar body adheres to the outside of the elevating fertilization envelope as the perivitelline space widens (Figs. 6–8). The videotapes also demonstrate that egg diameter may sometimes shrink slightly (at most by 5 μm) as elevation of the fertilization envelope begins; however, such shrinkage does not always take place. In the first few minutes after insemination, eggs remain nearly spherical and undergo deformations that are at most slight (compare Figs. 6 and 7) if detectable at all. In the lancelet, fertilization is not followed by conspicuous deformations comparable to those described for some ascidian eggs (Sawada, 1988). In the eggs of *B. floridae*, the most marked change in egg shape is a little flattening at the animal pole during the emission of the second polar body approximately 8 min after insemination. In the present study, we made no observations on living eggs before spawning; therefore, it remains possible that there are additional, very early episodes of egg deformation—either before or during first polar body emission.

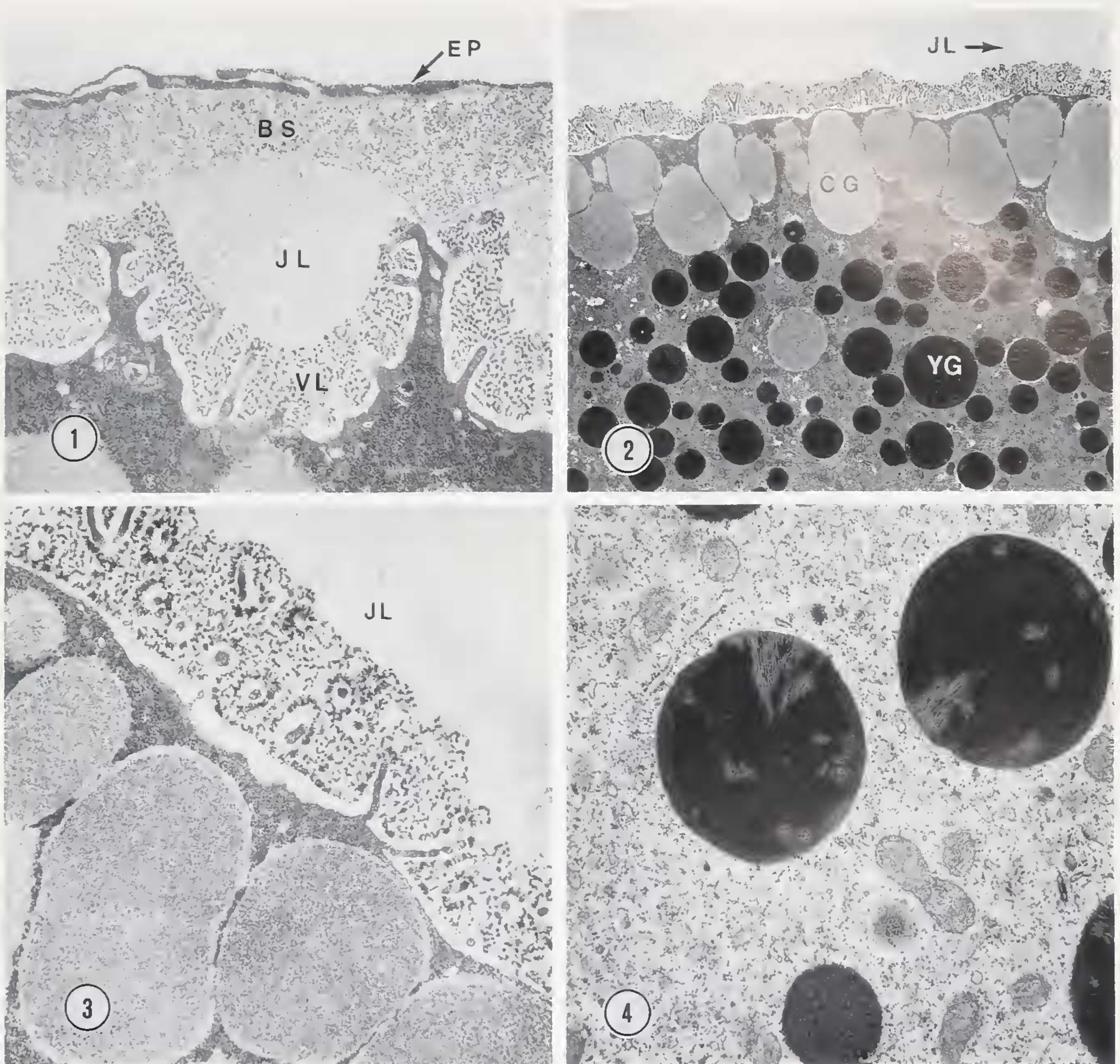


Figure 1. TEM of the ripe ovary of a lancelet. The periphery of a large oocyte (at bottom with pitted surface topography) is surrounded by a vitelline layer (VL) and, external to that, a jelly layer (JL). The associated ovarian tissues are the blood sinus (BS) and the epithelium lining the primary ovarian cavity (EP), which are left behind in the ovary when the oocyte along with its vitelline layer and jelly layer are ovulated. ×18,000.

Figure 2. TEM of a lancelet egg that has been spawned but not fertilized. The jelly layer (JL) is patchily distributed and adheres to the outside of the vitelline layer. Most of the egg cortex is occupied by a monolayer of cortical granules (CG), and yolk granules (YG) are the most conspicuous organelles deeper in the cytoplasm. ×3000.

Figure 3. TEM of the periphery of a lancelet egg that has been spawned but not fertilized. The jelly layer (JL) overlies the vitelline layer, and cortical granules are the predominant organelle in the egg cortex (toward the lower left). ×18,000.

Figure 4. TEM of the deeper cytoplasm of a lancelet egg that has been spawned but not fertilized. There are numerous free ribosomes, mitochondria, and yolk granules. Near the top left, there is a profile of rough endoplasmic reticulum. ×18,000.

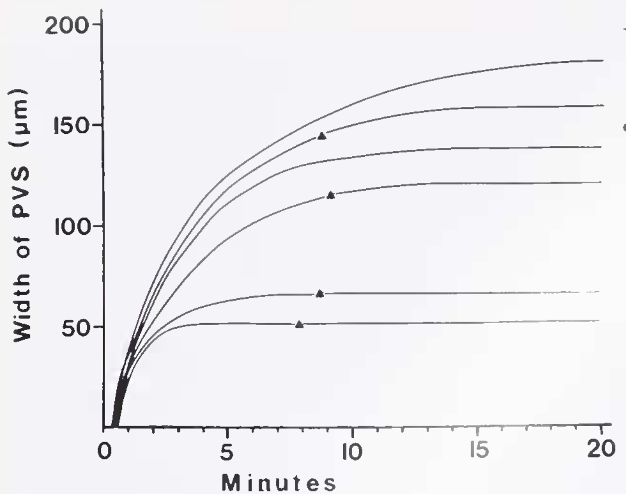


Figure 5. The time course of the elevation of the lancelet fertilization envelope. For six eggs, the width of the perivitelline space (PVS) is plotted against time in minutes after insemination. The data were gathered from video recordings made through the LM. For four of the eggs, triangles mark the time of second polar body emission (the other two eggs were unfavorably oriented and the second polar body was not visible). The filled circle shows the mean width of the perivitelline space of 43 eggs measured 20 min after insemination; the error bars are ±1 standard deviation.

For most eggs, the maximum width of the perivitelline space (147 μm; SD = 46; n = 43) is reached by 20 min after insemination. In spite of considerable scatter in the data, there is a significant ($P = 0.008$) correlation between egg diameter and width of the perivitelline space 20 min after insemination (Pearson product moment statistic = 0.401; $r^2 = 0.141$). The regression is: space width = egg diameter × 2.03 − 128.7. The mean width of the perivitelline space just before hatching (9 h after insemination) is 165 μm (SD = 24; n = 10). Due to the large standard deviations, there is no significant difference between the mean widths at 20 min and 9 h.

Cortical reaction 40 s after insemination (TEM Figs. 9–10)

In *B. floridae* the exocytosis of the cortical granules takes place over the entire egg surface almost simultaneously: there is no definite progression of the cortical reaction in a wave sweeping over the egg surface such as in echinoids. Scattered cortical granules may briefly lag behind their neighbors in undergoing exocytosis, but within a few seconds the egg cytoplasm contains virtually no unreacted cortical granules. At exocytosis, the cortical granule membrane fuses with the overlying plasma membrane, and the granule contents swell and appear as a mass of loosely packed granular and fibrillar material. As the cortical granule material pushes the vitelline layer away from the egg surface, the vitelline layer becomes thinner (reduced to only 0.3 μm in some places). Moreover, another layer (Fig. 10, arrows), previously undescribed, forms where cortical granule material comes into contact with the inside of the vitelline layer. We will call this new layer, which is about 20 nm thick and has a dense, very finely granular consistency, the *inner layer of the fertilization envelope:* from this time in development, we will call the vitelline layer the *outer layer of the fertilization envelope.* It is likely that the inner layer forms from cortical granule material, although this point cannot be proven unequivocally from static morphological data. The thin jelly layer is still present on the surface of the fertilization envelope.

Completion of the hyaline layer 80 s after insemination (TEM Figs. 11–12)

At 80 s after insemination, most of the cortical granule material expelled into the perivitelline space has become confluent to form a hyaline layer, which was first described from LM sections by Sobotta in 1897 (see Table 1). This layer is conspicuous in TEM, although almost invisible in LM of living material. The recently formed hyaline layer has an average thickness of roughly 10 μm and is composed of a diffuse, finely fibrogranular material with a low electron density. Scattered within the hyaline layer, there are some membranous profiles that are probably the remains of microvilli torn away from the egg surface during the cortical reaction. The space between the inner surface of the hyaline layer and egg plasma membrane also contains abundant microvilli, at least some of which are still part of the egg surface. Peripheral to the hyaline layer, the fertilization envelope consists of a 200 nm thick outer layer (derived from the vitelline layer) and a 30 nm thick inner layer (evidently derived from cortical granule material). There is no longer any trace of the jelly layer that was formerly located on the surface of the fertilization envelope.

Fertilization envelope and hyaline layer up to hatching (TEM Figs. 13–14)

During the first few minutes after insemination, not only the fertilization envelope, but also the hyaline layer elevates from the plasma membrane of the fertilized egg. In not remaining closely associated with the egg surface, the hyaline layer resembles that of ophiuroids (Holland, 1979) and not that of echinoids (Chandler and Heuser, 1980; Cameron and Holland, 1985).

Both the hyaline layer and the outer layer of the fertilization envelope rapidly become thinner as the perivitelline space widens. By 20 min after insemination, the hyaline layer has a width of 1 μm or less, and the outer layer of the fertilization envelope is reduced to about 25 nm

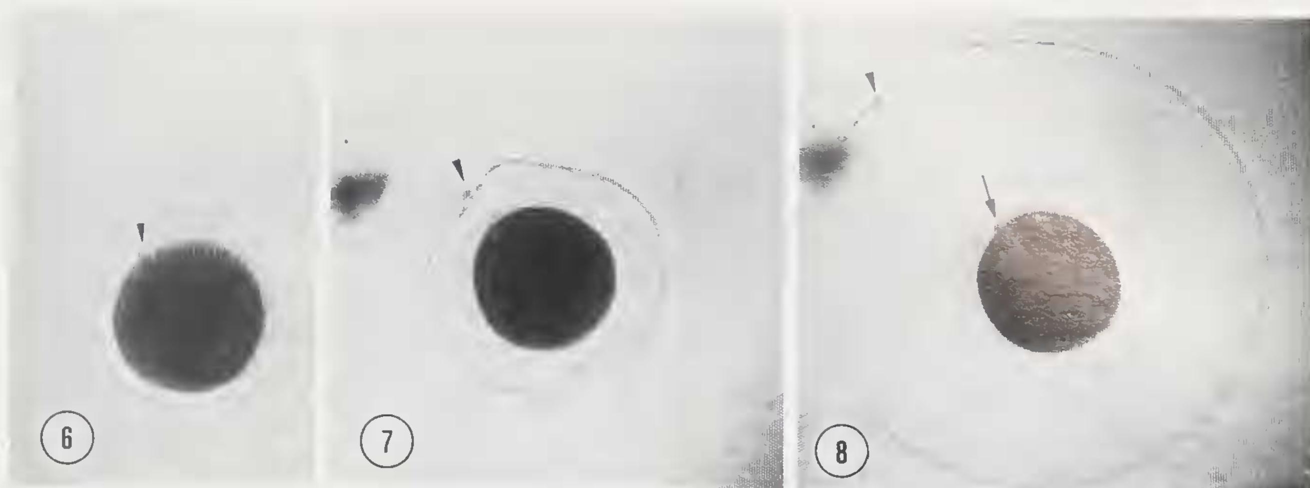


Figure 6. Videotape LM of a lancelet egg that has been spawned but not fertilized; the arrowhead marks the first polar body. ×140.

Figure 7. Videotape LM of a lancelet egg 80 s after insemination. The first polar body (arrowhead) adheres to the outside of the rising fertilization envelope. ×140.

Figure 8. Videotape LM of a lancelet egg 20 min after insemination. The first polar body (arrowhead) adheres to the fully risen fertilization envelope, and the second polar body (arrow) adheres to the animal pole of the egg. ×140.

and may be interrupted in some places. It is not clear whether such interruptions are due to abrasion or extreme stretching (by 20 min after insemination, the surface area of the risen fertilization envelope is about ten times its original surface area). In contrast, the inner layer of the fertilization envelope is complete and, if anything, even thicker (45 nm) than it was previously. It is possible that part of the substance of the hyaline layer is continuously added to the fertilization envelope's inner layer to maintain the integrity of the latter. As the time of hatching approaches (about 9 h after insemination), the thickness of the hyaline layer has been reduced to about 150 nm, due perhaps to the influence of the embryo's ciliated epidermis or to a hatching enzyme secreted by the embryo. Moreover, the outer layer of the fertilization envelope is completely absent in many places. Apparently, the chief barrier that the embryo must pass through at hatching is the inner layer of the fertilization envelope.

Discussion

Possible functions of lancelet egg coats

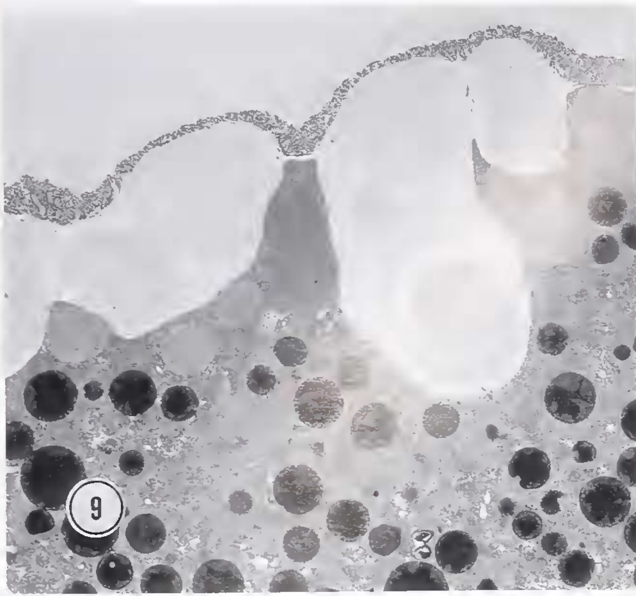
The jelly layer and/or the vitelline layer of the unfertilized lancelet egg probably interact with approaching sperm to trigger the acrosome reaction and then serve as sites for the initial binding of the sperm. Sobotta (1897) shows sperm bound to the elevating fertilization envelope of a lancelet in his LM illustrations, but a thorough TEM study of sperm binding and entry is needed.

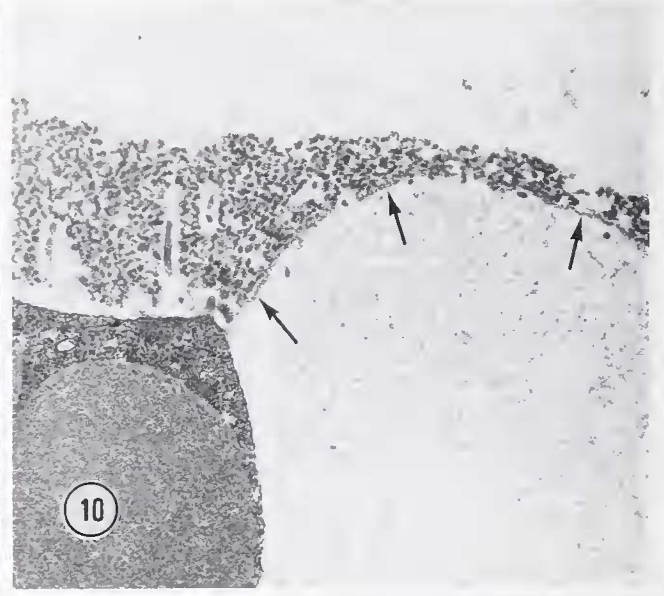
Although nothing definite is known about polyspermy blocks in lancelets, it may well be that the formation of the fertilization envelope establishes a slow, permanent block following a fast, transient block due to a fertilization potential at the level of the plasma membrane. Such a dual system to ensure monospermic fertilization is well known in some other deuterostomes (reviewed in Mazzini *et al.*, 1984, and Elinson, 1986).

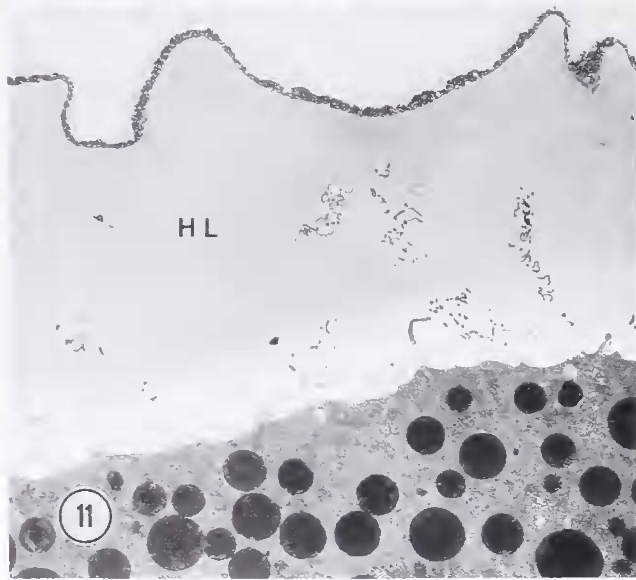
Removal of both the fertilization envelope and the hyaline layer has no adverse effect on lancelet development (Tung *et al.*, 1958), and thus these egg coats are not required for normal morphogenetic movements (*e.g.*, gastrulation) in laboratory culture. In the natural environment, however, the egg coats probably play a role in protecting the developing embryos from mechanical damage, microbial attack, and predation. Our incidental observations on living and fixed material have shown that eggs and embryos within an elevated fertilization envelope sink much more slowly than unfertilized eggs. This phenomenon, which deserves more careful study, should have important consequences for the bathymetric distribution and lateral transport of lancelet embryos in nature.

Comparison of egg coats among deuterostomes

We will limit this discussion to deuterostomes in a restricted sense (*i.e.*, as comprising the echinoderms, hemichordates, and chordates, but excluding the enigmatic chaetognaths). Readers wishing a review of egg coat evo-







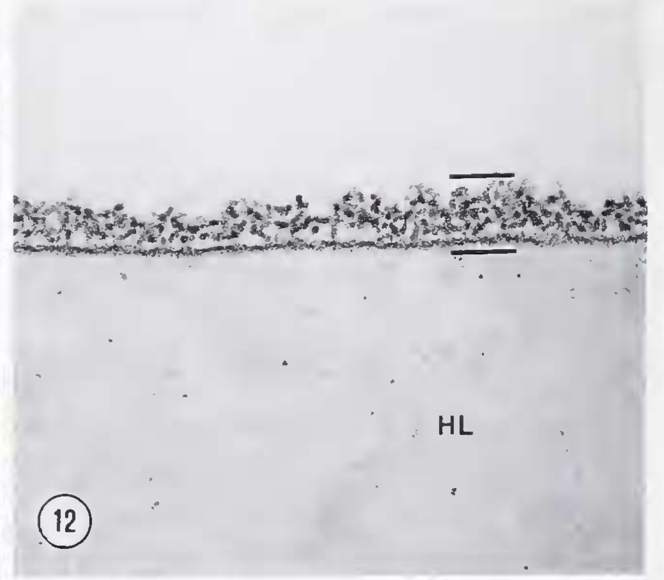


Figure 9. TEM of a lancelet egg 40 s after insemination while the cortical reaction is in progress. Important features from top to bottom are: the inconspicuous jelly layer, the vitelline layer, ejected contents of cortical granules in the perivitelline space, and the egg cytoplasm still containing some unreacted cortical granules and numerous yolk granules. ×3000.

Figure 10. TEM of the periphery of a lancelet egg 40 s after insemination. The inconspicuous jelly layer (at top) overlies the vitelline layer, which is being converted into the fertilization envelope by the

lution for the entire animal kingdom are directed to Mazzini *et al.* (1984). The present discussion will largely exclude homologies among the ovarian layers, because their embryonic origins and fine structures are in need of further study in some lower deuterostomes: thus we will concern ourselves chiefly with the coats surrounding the spawned egg before and after fertilization. These egg coats usually have little structural complexity even as seen by TEM, and, when such uncomplicated structures are compared, homoplasies are most easily mistaken for homologies (Ruppert, 1982). Therefore, the homologies connoted by our terminology should remain tentative until additional information (especially on the detailed biochemistry) is at hand. Unless explicitly stated below, the references are for species that spawn their eggs freely into the water.

Hemichordates and echinoderms compared to lancelets. Before fertilization, the coats of the spawned lancelet egg have most in common with hemichordate and echinoderm eggs. The coat just outside the egg plasma membrane is the vitelline layer, and the coat just outside the vitelline layer is the jelly layer in enteropneust hemichordates (Hadfield, 1975), in crinoids (Holland, 1977) in ophiuroids (Holland, 1979; Yamashita, 1984), in echinoids (numerous references, the most comprehensive being Chandler and Heuser, 1980), and in asteroids (Holland, 1980; Longo *et al.*, 1982; Souza and Azevedo, 1988). In comparison to echinoderms, lancelets have a much thicker vitelline envelope with a more coarsely granular structure. The initial thickness of the layer in lancelets may be related to the great attenuation it must undergo during the enormous expansion of the perivitelline space after fertilization.

As in lancelets, the fertilized eggs of the hemichordates and echinoderms mentioned in the previous paragraph undergo cortical granule exocytosis, and some of the cortical granule contents become added to the vitelline layer to produce a composite structure, the fertilization envelope. The hyaline layer, which forms in the perivitelline space from other components of the cortical granules, has been described only for the lancelet and for some echinoderms (namely, echinoids, ophiuroids, and, more arguably, asteroids). However, for enteropneust hemichordates, existing TEM studies are not adequate to establish whether a hyaline layer is present, and further work is needed.

The unfertilized eggs of some hemichordates and a few echinoderms may lack egg coats present in unfertilized lancelet eggs. Pterobranch hemichordates appear to have no egg coats of any kind (Lester, 1988), perhaps because they brood their eggs and embryos, and no vitelline layer can be demonstrated in holothurians (Holland, 1981).

Tunicates (phylum Chordata: subphylum Urochordata) compared to lancelets. Among the tunicates, appendicularians have egg coats most similar to lancelet egg coats (Holland *et al.*, 1988). The unfertilized appendicularian egg is surrounded by a vitelline layer, but lacks a jelly layer. There are no follicle cells outside the vitelline layer in several species of appendicularians, and never any test cells beneath it. After fertilization, appendicularian eggs undergo cortical granule exocytosis, and some of the contents of these organelles ultimately adhere to the vitelline layer. However, no trace of a hyaline layer ever forms.

Other tunicates, whether they brood their eggs or spawn them freely in the water, have some of the most unusual egg coats in the animal kingdom. The vitelline layer (often called the chorion in the ascidian literature) is underlain by scattered test cells and overlain by a layer of follicle cells (Berrill, 1975; Lambert and Koch, 1988). Moreover, the egg has no jelly layer. After fertilization, there are no cortical granules in the egg to undergo exocytosis, and the egg coats, as seen by TEM, remain virtually unchanged. Therefore, the egg coats of tunicates

addition of a thin inner layer (arrows) to its inner surface. This inner layer of the fertilization envelope is probably derived from part of the ejected cortical granule contents. An unreacted cortical granule remains in the cortical cytoplasm at the lower left. ×18,000.

Figure 11. TEM of a lancelet egg 80 s after insemination. The jelly layer has disappeared, and the fertilization envelope (at top) is elevating from the surface of the egg (at bottom). Much of the intervening perivitelline space is occupied by the hyaline layer (HL) formed from the ejected contents of the cortical granules. The membranous profiles in the perivitelline space are probably microvilli torn away from the egg surface during the cortical reaction. ×3000.

Figure 12. TEM of the lancelet fertilization envelope (between the two parallel lines) 80 s after insemination. The thicker outer layer is derived from the former vitelline layer, and the thin inner layer is evidently derived from cortical granule material. The outer part of the hyaline layer (HL) appears in the bottom half of the figure. ×30,000.

Figure 13. TEM of the lancelet fertilization envelope 20 min after insemination. The outer layer of the fertilization envelope and the hyaline layer (HL) are both much thinner than before. ×30,000.

Figure 14. TEM of the lancelet fertilization envelope 8 h after insemination (1 h before hatching). Only scattered fragments of the outer layer of the fertilization envelope remain, and the hyaline layer (HL) is relatively inconspicuous. ×30,000.

other than appendicularians are very different from those of lancelets. Unfortunately, Reverberi (1966, 1971) had a strongly preconceived notion that lancelet eggs should resemble ascidian eggs, and he was led into making some egregiously incorrect homologies, as Guraya (1978, 1983) has justly pointed out. Reverberi, in the first TEM studies of lancelet oocytes in the ovary, identified the vitelline layer as the equivalent of the test cells in ascidians, and he identified the ovarian blood sinus as a layer of follicle cells: in truth, neither the vitelline layer nor the blood sinus of lancelets is a cellular structure at any time during oogenesis. In 1972, Reverberi and De Leo gave the structures in question less definite names (Table I), but by then the original misinterpretations were well entrenched in the literature.

Lower vertebrates (phylum Chordata: subphylum Vertebrata) compared to lancelets. Guraya (1978, 1983), in LM studies of lancelet ovaries, believed that the entire blood sinus (that is the blood plasma and its bounding basal laminae) was a single, very thick basal lamina. To Guraya, such a basal lamina appears homologous to the one separating the follicle from the theca in vertebrate ovaries, and his names for the other investments of the oocytes (Table I) are also taken from vertebrate ovarian histology. In short, he considers the ovarian oocyte of a lancelet to be like that of a vertebrate without its follicle cell layer. We partially agree with Guraya's implied homology between the vertebrate zona pellucida and the lancelet vitelline layer. However, it is likely that the lancelet vitelline layer is a primary envelope, while the zona pellucida of lower vertebrates (cyclostomes and fishes) is a compound envelope (*sensu* Wourms, 1987), having been produced by both the oocyte and the follicle cells (Hardisty, 1971; Laale, 1980; Dumont and Brummett, 1985). Any jelly coating the outside of the zona pellucida of vertebrate eggs is secreted by the oviduct and is thus not homologous to the jelly layer of lancelets, hemichordates, and echinoderms.

Fertilization in the vertebrate egg often results in cortical granule discharge (although not in elasmobranchs, urodeles or birds, which lack such organelles). A few TEM studies of fertilized eggs of vertebrates have indicated that cortical granule material is added to the zona pellucida (= vitelline layer); *i.e.*, in anurans the added material evidently forms the F layer (Grey *et al.*, 1974). In most vertebrates, however, such additions are inconspicuous if detectable at all by TEM (Dumont and Brummett, 1985). Finally, structures comparable to the hyaline layer of lancelets and some echinoderms have almost never been found in the perivitelline space around any vertebrate egg; the only possible hyaline layer homologue described to date for any vertebrate is the S layer surrounding the fertilized anuran egg (Larabell and Chandler, 1988a, b).

Implications of the present study for lancelet phylogeny

The new information in the present paper is not extensive enough to warrant a full review of the many proposed schemes of lancelet phylogeny. Even so, two of these schemes can profitably be re-examined in the light of our results. At the outset, we should state that we think the hemichordates and echinoderms are appropriate outgroups for analyzing relationships within the chordates.

We will consider phylogenetic scenarios deriving lancelets from tunicate-like antecedants, since we favor the commonest cladistic arrangement of the chordate subphyla: namely that the tunicates are the sister group of the lancelets plus vertebrates (Hennig, 1983; Ghiselin *et al.*, 1986). By outgroup comparison, all the character states of the lancelet egg coats are plesiomorphic for the phylum Chordata. This means that any group interposed between the lancelets and the base of the phylum Chordata should have egg coats with as few apomorphies as possible.

The appendicularian tunicates have fewer apomorphies (the loss of the jelly layer and the loss of the hyaline layer) than the ascidian and thaliacian tunicates, which have the following apomorphies: loss of the jelly layer, loss of the hyaline layer, loss of cortical granules, addition of test cells, and addition of follicle cells. If one assumes that the stem tunicate was an appendicularian-like animal with eggs like modern appendicularians, then lancelets derived from it need only regain the jelly layer and hyaline layer. The alternative that eggs of the appendicularian-like stem tunicate had not yet lost the jelly and hyaline layers, would mean that lancelet eggs would not have had to regain any structures. In contrast, if one assumes that the stem tunicate was an ascidian-like animal with eggs like those of modern ascidians, then lancelets derived from it must lose test cells and follicle cells while regaining the cortical granules, jelly layer and hyaline layer. Thus, from a consideration of egg coats in deuterostomes, we suggest that the ancestral chordate resembled an unspecialized appendicularian (a theory best developed by Brooks in 1893) and was not like an adult ascidian that developed a tadpole larva which became neotenous and gave rise to lancelets and vertebrates (a theory best developed by Garstang in 1928).

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